

Influence of Substrate on Behavior of a Cichlid Fish

Undergraduate Research Thesis

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by

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Abstract

The purpose of this experiment is to determine how substrate type influences behavior in a group-living cichlid fish (*Neolamprologus pulcher*). Preliminary observations suggest a decrease in reproductive success of lab-reared fish living in tanks with darker substrate. I hypothesize that substrate type influences behaviors involved in reproduction, such as mating displays and maintenance of breeding shelters. Therefore, I predicted that fish in tanks with light colored substrate would exhibit higher frequencies of such behaviors as compared to tanks with darker substrate. To test this, I established 10 groups of three fish each. The substrate in five of the tanks was light-colored substrate; substrate was dark in the other five. Over a period of 10 weeks, two rounds of 15 min behavioral observations were conducted per tank each week using the event-recorder software, Boris. I examined investment in breeding shelter maintenance by filling these with sand and measuring the rate at which fish removed sand over a 4-week time period. Generalized Linear Mixed Models (GzLMM) were used to test the effects of treatment and actor, and the interaction between these, on social behaviors and interactions with substrate. This study will contribute to understanding the relationship between habitat composition and behavior in fish.

Introduction

The abiotic environment influences the social behaviors of organisms (Hamby et al 2016). For many species, abiotic factors such as temperature or visual clarity can influence reproductive behaviors associated with productivity, such as courtship and communication between individuals (Hamby et al 2016; Steele and Lairdre, 2019). In an evolutionary context, abiotic factors in the environment act as selective pressures on behavior, playing a critical role in the social evolution in organisms (Steele and Lairdre, 2019). Ecological constraints, or the

abiotic factors in the environment that impact social behavior are not well studied in the literature (Steele and Laidre, 2019). Thus, this study seeks to increase our understanding of the abiotic environment and how it influences social behaviors in organisms, specifically in a group-living cichlid fish, *N. pulcher*. Cichlid fish are a highly studied group that has fascinated researchers due to the species incredible diversity and evolutionary history (Kornfield and Smith, 2000). Group-living *N. pulcher* are characterized by having complex social behaviors and social structures (Koblmuller et al 2019).

As a substrate brooding fish, breeding substrate is a critical habitat component in the breeding success of *N. pulcher*. Substrate can influence behavior and the structure of dominance hierarchies in cooperative breeding systems (Josi et al 2018). For example, competition for substrate between conspecifics for suitable breeding habitat in the cichlid fish, *Lamprologus callipterus*, can result in aggressive behaviors between fish, and in extreme cases can result in infanticide and death of individuals (Maan & Taborsky, 2007). Substrate also provides necessary protection from predator species and provides important sites for reproduction (Hellmann & Hamilton, 2018). The potential for substrate to influence social environment of *N. pulcher* raises questions for researchers aiming to understand the importance of habitat suitability on the breeding success of *N. pulcher*. As a result, behaviors associated with the substrate in both male and female fish can provide us insight into how habitat may be playing a role in reproductive success.

In *N. pulcher*, breeding shelter maintenance is utilized to establish breeding sites (Josi et al 2018). Dominant fish will dig and remove substrate and lay their eggs on the underside of large rocks (Josi et al 2018). Thus, having high quality suitable substrate plays an important role in the reproductive success in *N. pulcher*. If suitable substrate is not available and habitat

provided for housed lab fish is considered poor, fish will ultimately suffer and fail to be reproductively productive (e.g., *Oreochromis mossambicus*, Galhardo et al 2009). This poses to be a critical issue for researchers seeking to study this model organism; and also results in an overall decreased quality of life for the fish reared in a lab setting.

The purpose of this experiment is to determine how substrate type influences behavior in a group-living *N. pulcher*. Preliminary observations suggest a decrease in reproductive success of lab-reared fish living in tanks with darker substrate (Hamilton, pers. obs.). I hypothesized that substrate type influences behaviors involved in reproduction, such as mating displays and maintenance of breeding shelters. Therefore, I predicted that fish in tanks with light colored substrate would exhibit higher frequencies of such behaviors as compared to tanks with darker substrate.

Much of the literature has addressed preference of substrate by individual fish, providing choices measured by time individual fish has spent in each substrate type (Galhardo et al 2009; Josi et al 2019). Rather than seeking to evaluate preference of individual fish, this study seeks to observe various reproductive behaviors in established social groups of *N. pulcher*. By comparing how social interactions vary between two different substrate habitats (dark and light substrate), potential interactions between actors within the social group and the type of treatment (substrate) that composed each individual group. Additionally, by manipulating conditions within tanks, I can induce breeding shelter maintenance within these established social groups, and subsequently observe the behavioral responses of fish within different substrate habitats.

Methods

To test the influence of substrate on the behavior of *N. pulcher*, I established 10 groups of three fish each. Groups were introduced to the study tanks around a month prior to the start of the study, to ensure there were no external effects as a result of group establishment prior to conducting behavioral observations. The groups were composed of a large, dominant female, a large, dominant male, and a smaller subordinate male or female. The dominant males were larger in size than the dominant females, in order to avoid conflict between members within the tank (Ligocki et al 2015). In order to distinguish individuals within the groups, each individual fish had elastomer markings, and the dominant females in each group had their dorsal fins clipped. The clipped fins allowed for easy identification of the dominant female within the group, and was repeated twice throughout the study. If an individual in a group exhibited aggression towards one of the members in the tank, the aggressor was immediately isolated from the group and pulled from the study for a week.

All 10 tanks that housed the groups had ~3 inches of substrate at the bottom of the tank, with temperatures within the tank set to a standard 77-80 degrees Fahrenheit; the optimal tank conditions for *N. pulcher*. Each of the 30-gallon tanks consisted of 2 individual terracotta pots halves to serve as breeding structures, with small PVC piping sections that were attached to the top of the tank to serve as hiding structures for subordinate fish. All tanks were designed to be similar in condition, except for the type of sand that composed the substrate layer in each tank. The substrate in five of the tanks was light-colored CaribSea Instant Aquarium Tahitian Moon Reef & Marine Substrate, and the substrate was dark CaribSea Eco Complete Zack Black in the other five. Tanks were placed in rows on two lab tables within the lab, and tank order alternate between dark-colored and light-colored substrate.

To encompass potential breeding cycles of the groups, the study was conducted over a period of 10 weeks. Two rounds or 15 min observations were recorded each week for each of the 10 tanks. Order of tanks observations was selected using the random number generator in excel (Microsoft Corporation, Redmond, WA). Video data was collected using Nikon Coolpix waterproof digital camera and Cannon, providing high quality data for analysis (Nikon, Japan; Cannon, Tokyo). In order to record each tank, cameras were placed on tripods while a timer was set to record the 15-minute length. White plastic backgrounds were placed behind tanks to ensure visibility of each individual within the tank throughout the observations. Behavioral observations were then conducted for each tank over the 10-week study period using the event- recorder software, Boris. An existing ethogram was used to analyze the observational data (Ligocki et al 2015; Reddon et al. 2015; Sopinka et al. 2009; Table 1). An additional 4-week study was conducted to analyze investment in breeding shelter maintenance, with 2 observations per tank per week. By filling each individual breeding shelter entirely with the substrate and subsequently measuring the rate at which individual fish removed sand over a 4-week period; we were able to measure investment in breeding shelter maintenance. The rate of sand removal by fish was observed when fish would either carry sand out of the breeding structure or digging sand out of the structures. All behavioral observations within the 4-week study were also scored using Boris, to establish if there were any significant behaviors other than substrate interaction that may have resulted from filling terracotta pots with sand. The resulting data was exported from Boris and organized in excel for analysis in R.

Using the open source software, R-studio, the statistical analysis was conducted on the resulting behavioral observation data from both the observational 10-week study and the 4-week breeding maintenance experiment. Generalized Linear Mixed Models (GzLMM) were used to

test the effects of treatment and actor and the interaction between these, on social behaviors and interactions with substrate. Data were tested for overdispersion. If significant overdispersion was found, I used a negative binomial distribution and a log-link in the GzLMM; otherwise, I used a Poisson distribution and log-link. Utilizing the General Linear Mixed Models for data analysis controlled for the repeated collection of observational data on each tank. The actors were defined as individuals who performed behaviors; which included the dominant male, dominant female, and subordinate male. In order to conduct the analysis, the behaviors were subset into categories; these 4 types of behaviors included submissive, aggressive, affiliative, and substrate interactions (Table 1). Each guild of behaviors was subsequently tested to determine whether the data fit a poisson distribution, or if the distribution over dispersed. The significant interactions between treatment and actor for the substrate behaviors in both the 10-week observational study and 4-week breeding shelter maintenance were plotted (Figure 1; Figure 2).

Results

For the 10-week study observational data, the substrate behaviors like digging and carrying sand were frequent (substrate: $\bar{x} = 7.64$, $\sigma = 9.42$). Aggressive, affiliative, and submissive behaviors respectively were relatively less frequent in the 10-week observational study (aggressive: $\bar{x} = 1.66$, $\sigma = 1.36$; affiliative: $\bar{x} = 1.18$, $\sigma = 0.49$; submissive: $\bar{x} = 1.55$, $\sigma = 1.30$). Similarly, in the 4-week breeding maintenance study, substrate behaviors such as digging and carrying sand were frequent (substrate: $\bar{x} = 13.26$, $\sigma = 16.82$), while aggressive, affiliative, and submissive observations were less frequently observed (aggressive: $\bar{x} = 1.60$, $\sigma = 1.18$; affiliative: $\bar{x} = 1.20$, $\sigma = 0.41$; submissive: $\bar{x} = 1.45$, $\sigma = 0.97$).

In the Generalized Linear Mixed Models (GzLMM) for the 10-week observational study, there was a significant effect of the interaction between actor and treatment in respect to

substrate behaviors ($\chi^2 = 7.46$, $p < 0.05$,; Table 2). In the GzLMM in the 4-week breeding maintenance study, there was a significant effect of the interaction between the treatment and actor in respect to substrate behaviors within the 4-week breeding structure maintenance study ($\chi^2 = 13.92$, $p < 0.05$; Table 3). The models for both the 10 week and 4-week study did not indicate any other significant interactions.

I did not find a significant effect of treatment, or the interaction between actor and treatment on the frequency of aggressive behavior in 10-week observational study (aggressive: $\chi^2 = 5.31$, $p > 0.05$; Table 2). I also did not find a significant effect of treatment, or the interaction between actor and treatment, on affiliative and submissive behaviors respectively (affiliative: $\chi^2 = 0.58$, $p > 0.05$; submissive: $\chi^2 = 0.32$, $p < 0.05$). In the 4-week breeding maintenance study, I did not find a significant effect of treatment, or the interaction between treatment and actor with respect to aggressive behaviors (aggressive: $\chi^2 = 0.35$, $p > 0.05$). The affiliative behaviors also showed no significant interaction between actor and treatment (affiliative: $\chi^2 = 0.10$, $p > 0.05$). I additionally found no significant effects of the interaction between treatment and actor relative to submissive behaviors in the 4-week breeding maintenance study (submissive: $\chi^2 = 0.28$, $p > 0.05$).

The results of plotting the least square means error bar indicate significant differences among individual actors (dominant male, dominant female, subordinate male or female) and between treatments (light-colored sand, dark-colored sand) with respect to substrate interactions (digging, carrying sand) within the tank. Both plots show similar trends in the data. Both plots indicate the female exhibited a significantly higher count substrate interactions in tanks with the dark-colored substrate relative to the light-colored tanks (Figure 1; Figure 2). The plots also show that males had significantly higher counts of substrate interactions in the light-colored sand

as compared to the dark-colored sand (Figure 1; Figure 2). In regard to the subordinate fish, the plot shows the low-ranking individuals in the social groups exhibited high counts of substrate interactions in the dark-colored substrate, but had significantly less numbers of observations as compared to the dominant male and female (Figure 1; Figure 2). Aggressive behaviors were plotted for additional visualization of the data, but the GLMM showed no significant interactions (Figure 3).

Discussion

This study found that changes in substrate composition does impact the behavior of *N. pulcher*. The results of the study show significant interaction between treatment and actor in respect to substrate behaviors. Specifically, I found a significant influence of the environment on behaviors such as digging and carrying sand. This was not surprising, because substrate does play a critical role in the behavior of *N. pulcher* (Josi et al 2018).

I did not find an effect of substrate on aggressive behaviors or other social behavior in *N. pulcher*. Social behavior is highly influenced by the abiotic environment in organisms (Hamby et al 2016), and I expected social behaviors to be influenced by the type of substrate. Availability of breeding substrate can result in group conflict, offspring loss, and lack of protection from predator species (Hellman & Hamilton, 2018; Hellman et al 2016; Maan & Taborsky, 2007). Spatial distribution of breeding substrate has also been found to influence the breeding behaviors of *N. pulcher*, which may also include increasing conflictive behaviors between conspecifics (Hamilton & Heg, 2007). The results of this study suggest that the costs and benefits associated with removing substrate from breeding shelters differ with substrate type. Therefore, I expected to see significant interaction between actor and treatment with respect to aggressive behaviors. Though this is unexpected, this may be a result of the small group size and the variation of size

of individuals within the tank. If members in a group are homogeneous in size, groups are more likely to experience conflict between conspecifics (Ligocki et al 2015). Small groups sizes are more socially unstable than large group sizes, and typically larger groups of fish have larger sizes of fish (Ligocki et al 2015; Heg and Bachar, 2006). This may explain why individuals in a group of three did not exhibit significant influence of treatment and actor relative to aggressive behaviors. Also, throughout the experiment the protocol reduced aggressive behaviors between conspecifics by isolating dominant individuals, which would result in a decrease in conflict within the tank (Ligocki et al 2015).

I found that male and female fish differed in the effects of sand type on behavior. Dominant females increased digging rate in dark substrate, but dominant males reduced their digging rate (Figure 2). These results suggest that substrates may differ in the costs or benefits for fish to move. The dark and light substrate may have differed in grain size, so that the costs of carrying sand may differ. The light substrate may have also resulted in higher amounts of reflected light within the tank. Variation of light in the habitat has been found to significantly impact the reproductive behaviors in another African cichlid, *Astatotilapia burtoni* (Maruska and Fernald, 2010), therefore may be attributing to the differences we see behavior of individuals. Finally, the dark substrate also had magnetic properties, while the light sand did not (personal observation). The magnetic properties of the dark colored substrate may also influence the costs of removal, such as accumulation of individual particles causing large clumps of substrate. If costs of removal differ, then larger males may have less of a cost for moving substrate due to their larger size. Differences between sexes may also be a result of compensating between the dominant breeding pairs, with one sex increasing their breeding activity as the partner decreases.

The results of this study indicate that male and female *N. pulcher* differ in their response to differences in the substrate composition in which their breeding substrate occurs. Removing sand by carrying or digging is an important reproductive behavior in *N. pulcher* (Josi et al 2018), suggesting that costs or benefits of breeding shelter maintenance show variation between dominant males and females. Therefore, the results of this study further our understanding of how the physical environment, in this case, the characteristics of breeding substrate, play an important role in reproductive lifecycle and sex differences in these behaviors in this *N. pulcher*.

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Table 1: Ethogram of behaviors used in the observational scoring software, Boris (Ligocki et al 2015; Reddon et al. 2015; Sopinka et al. 2009).

Behavior Type	Behavior
Aggressive	Ram
	Bite
	Tail Beat
	Mouth fight
	S-Shaped Bend
	Slow Approach
	Fast Approach
	Head-Down Display
	Fin Raise
	Opercula Spread
	Head Jerk
Submissive	Flee
	Avoid
	Tail Quiver
	Hook Display
	Bump
Affiliative	Join
Substrate	Dig
	Carry Sand

Table 2: Effects in the generalized linear mixed models (GzLMM) in the 10-week Observational Study. Significance is observed in the substrate behaviors, for the interaction between actor and treatment.

Behaviors	GLzMM	χ^2	DOF	p value
Substrate	Actor * Treatment	7.4619	2	< 0.05
	Actor	18.7496	2	> 0.05
	Treatment	0.1677	1	< 0.05
Aggressive	Actor * Treatment	5.3079	2	> 0.05
	Actor	4.6687	2	> 0.05
	Treatment	0.7211	1	> 0.05
Affiliative	Actor * Treatment	0.5840	2	> 0.05
	Actor	0.1403	2	> 0.05
	Treatment	0.1173	1	> 0.05
Submissive	Actor * Treatment	0.3151	2	> 0.05
	Actor	6.0151	2	< 0.05
	Treatment	2.3595	1	> 0.05

Table 3: Effects in the generalized linear mixed models (GzLMM) in the 4-week breeding shelter maintenance study. Significance is observed in the interactions between actor and treatment for the substrate behaviors.

Behaviors	GLMM	χ^2	DOF	p value
Substrate	Actor * Treatment	13.9156	2	< 0.05
	Actor	21.2128	2	< 0.05
	Treatment	2.3142	1	> 0.05
Aggressive	Actor * Treatment	0.3478	2	> 0.05
	Actor	2.1783	2	> 0.05
	Treatment	2.2509	1	> 0.05
Affiliative	Actor * Treatment	0.1045	2	> 0.05
	Actor	0.6063	2	> 0.05
	Treatment	0.1432	1	> 0.05
Submissive	Actor * Treatment	0.2823	2	> 0.05
	Actor	3.0601	2	> 0.05
	Treatment	3.9445	1	< 0.05

Table 4: Descriptive statistics for count of observations for behaviors in the observational 10-week study.

Behavior	Mean	Med	SD
Sum	3.519	1	6.102095
Substrate	7.638522	4	9.417629
Aggressive	1.664	1	1.357494
Affiliative	1.177778	1	0.4903101
Submissive	1.548507	1	1.30164

Table 5: Descriptive statistics for count of observations for the breeding shelter maintenance, 4-week study.

Behavior	Mean	Med	SD
Sum	5.9133	2	11.76517
Substrate	13.26289	8	16.81746
Aggressive	1.598958	1	1.175936
Affiliative	1.2	1	0.4140393
Submissive	1.449153	1	0.9748763

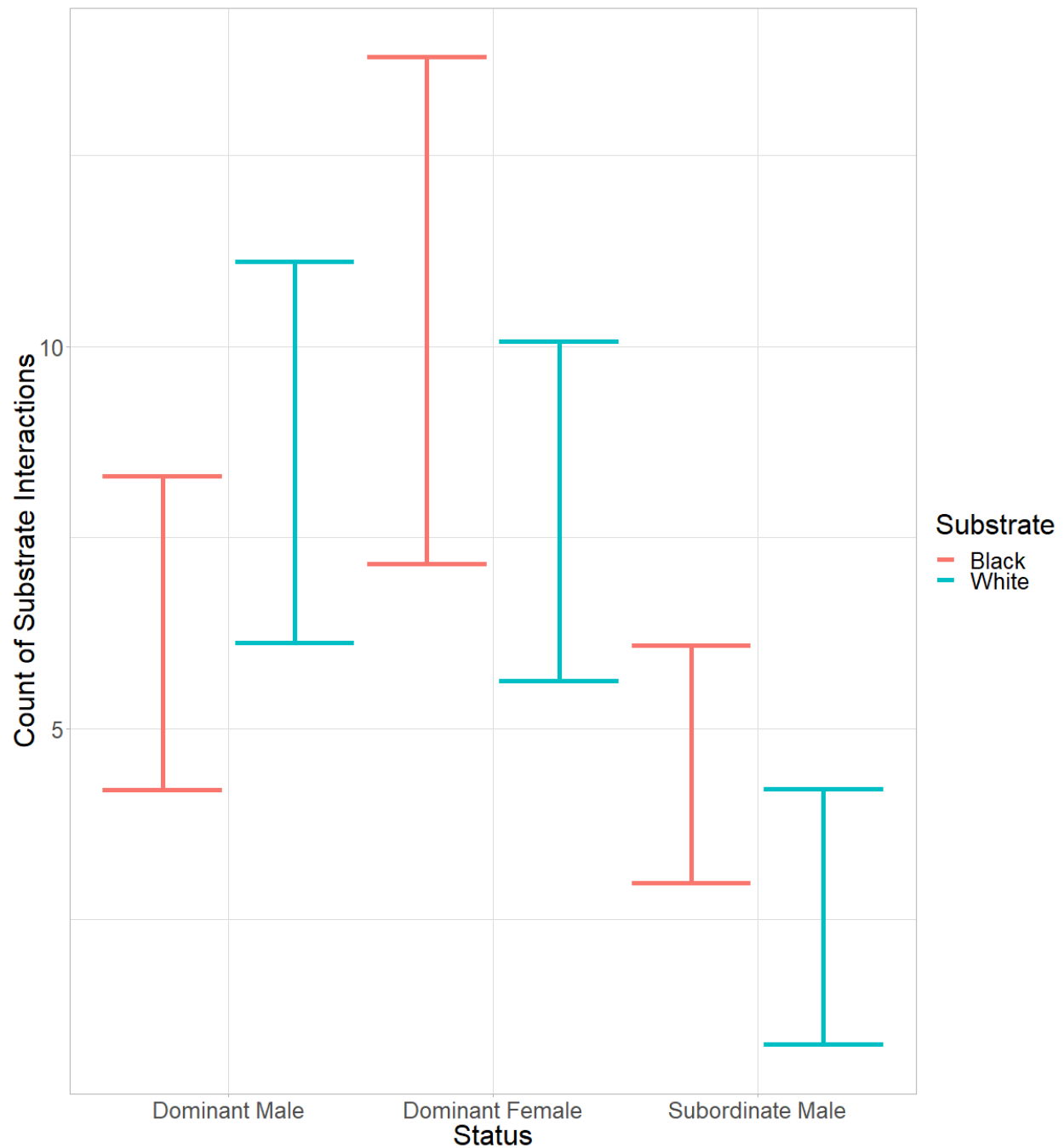


Figure 1: Dominant females exhibited significantly more digging behaviors in the black substrate as compared to the white substrate in the 10-week observational study. The dominant males exhibited significantly fewer substrate interactions in white substrate over black substrate.

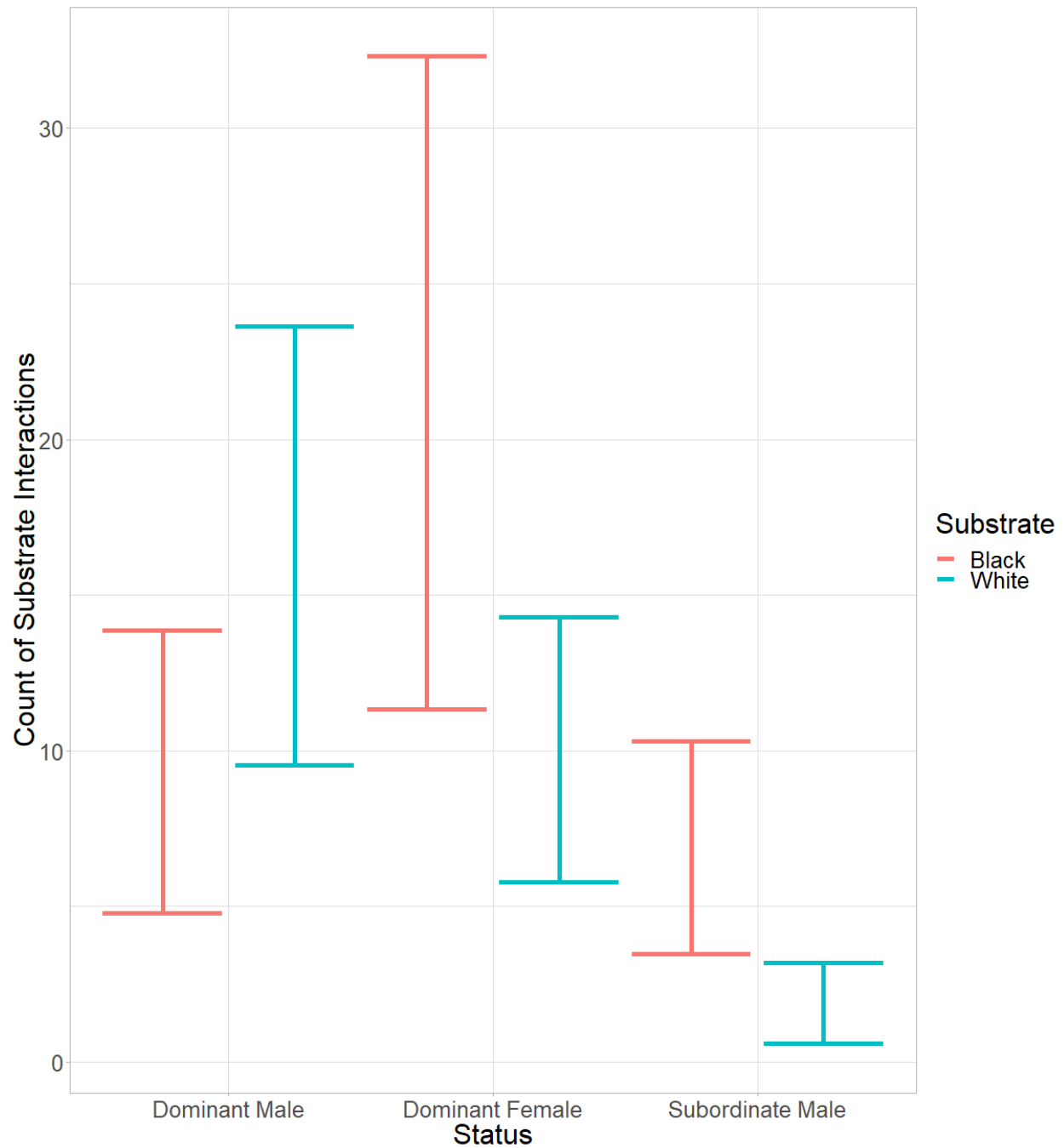


Figure 2: Dominant females exhibited significantly more digging behaviors in the black substrate as compared to the white substrate in the 4-week digging experiment. The dominant males exhibited significantly fewer substrate interactions in white substrate over black substrate.

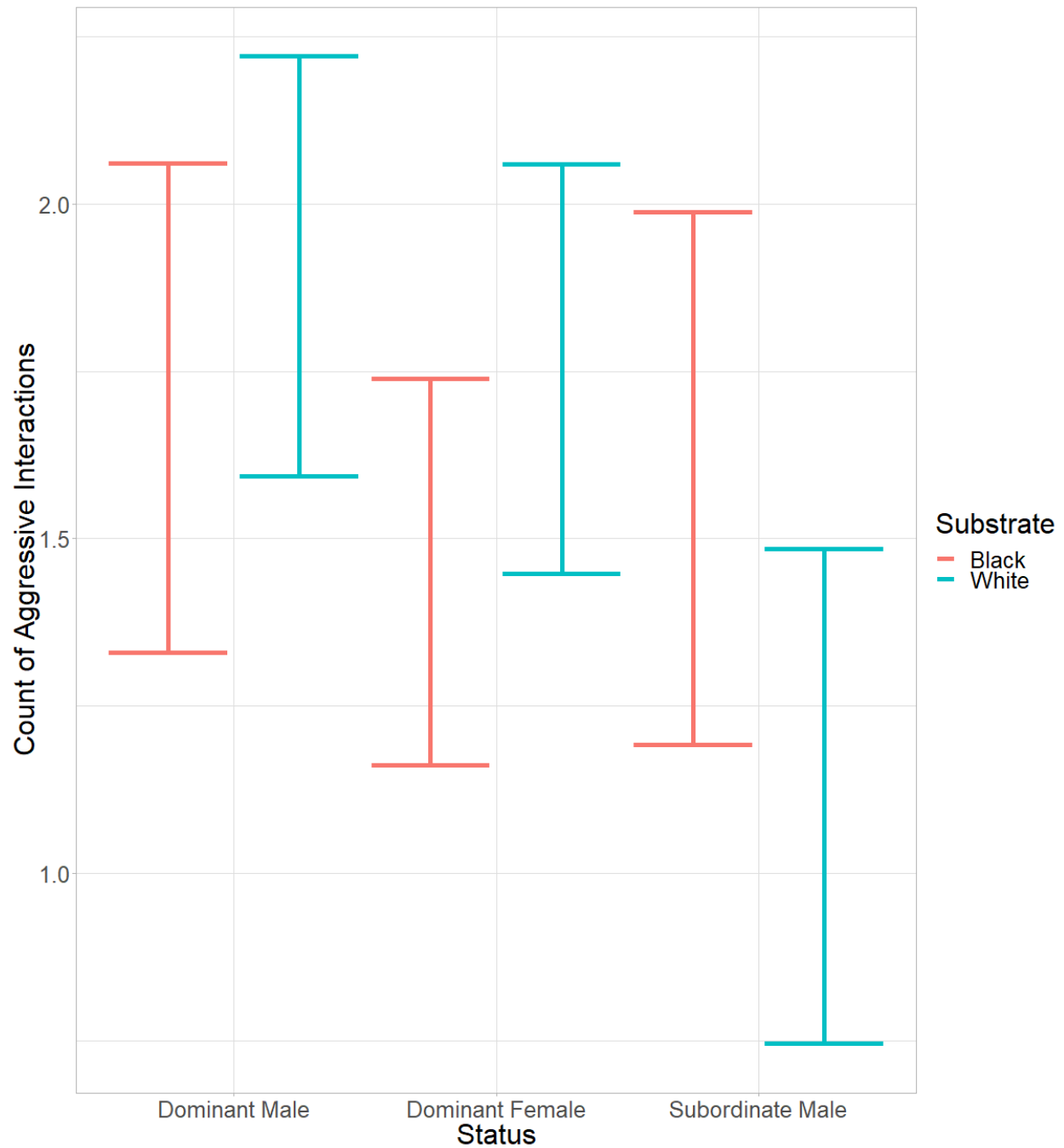


Figure 3: Aggressive interactions by actor status and substrate type. There were no significant effects of status, substrate or the interaction between these on the frequency of aggressive interactions.